PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : G01N 33/543, 33/53

A1

(11) International Publication Number: WO 97/29373

(43) International Publication Date: 14 August 1997 (14.08.97)

(21) International Application Number: PCT/FI97/00059

(22) International Filing Date: 4 February 1997 (04.02.97)

(30) Priority Data:
960534 6 February 1996 (06.02.96) FI

(71) Applicants *(for all designated States except US)*: WALLAC OY [FI/FI]; P.O. Box 10, FIN-20101 Turku (FI). ORION-YHTYMÄ OY [FI/FI]; P.O. Box 65, FIN-02101 Espoo (FI).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: SAVIRANTA, Petri [FI/FI]; Kreetankuja 9, FIN-21200 Raisio (FI).

(74) Agent: TURUN PATENTTITOIMISTO OY; P.O. Box 99, FIN-20521 Turku (FI).

(81) Designated States: JP, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: NON-COMPETITIVE IMMUNOASSAY WITH BLOCKING OF UNOCCUPIED SPECIFIC BINDING SITES ON SOLID PHASE

(57) Abstract

The present invention relates to a non-competitive method for the determination of analytes. Initially the analyte is bound to a specific binding partner, after which the unoccupied binding sites of the binding partner are inactivated. The bound analyte is then dissociated from the binding partner and replaced by a labelled marker, after which the bound labelled marker is determined. The signal from the bound labelled marker is directly proportional to the initial amount of analyte in the sample, which makes the present method more favourable than the competitive assays.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	**GB**	United Kingdom	**MW**	Malawi
AT	Austria	**GE**	Georgia	**MX**	Mexico
AU	Australia	**GN**	Guinea	**NE**	Niger
BB	Barbados	**GR**	Greece	**NL**	Netherlands
BE	Belgium	**HU**	Hungary	**NO**	Norway
BF	Burkina Faso	**IE**	Ireland	**NZ**	New Zealand
BG	Bulgaria	**IT**	Italy	**PL**	Poland
BJ	Benin	**JP**	Japan	**PT**	Portugal
BR	Brazil	**KE**	Kenya	**RO**	Romania
BY	Belarus	**KG**	Kyrgystan	**RU**	Russian Federation
CA	Canada	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CF	Central African Republic			**SE**	Sweden
CG	Congo	**KR**	Republic of Korea	**SG**	Singapore
CH	Switzerland	**KZ**	Kazakhstan	**SI**	Slovenia
CI	Côte d'Ivoire	**LI**	Liechtenstein	**SK**	Slovakia
CM	Cameroon	**LK**	Sri Lanka	**SN**	Senegal
CN	China	**LR**	Liberia	**SZ**	Swaziland
CS	Czechoslovakia	**LT**	Lithuania	**TD**	Chad
CZ	Czech Republic	**LU**	Luxembourg	**TG**	Togo
DE	Germany	**LV**	Latvia	**TJ**	Tajikistan
DK	Denmark	**MC**	Monaco	**TT**	Trinidad and Tobago
EE	Estonia	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**UG**	Uganda
FI	Finland	**ML**	Mali	**US**	United States of America
FR	France	**MN**	Mongolia	**UZ**	Uzbekistan
GA	Gabon	**MR**	Mauritania	**VN**	Viet Nam

NON-COMPETITIVE IMMUNOASSAY WITH BLOCKING OF UNOCCUPIED SPECIFIC BINDING SITES ON SOLID PHASE

This invention relates to a method for the non-competitive determination of analytes.

BACKGROUND OF THE INVENTION

The publications and other materials used herein to illuminate the background of the invention, and in particular, cases to provide additional details with respect to the practice, are incorporated by reference.

The application of biospecific binding partners for the determination of analytes from complex samples has gained widespread use in *in vitro* diagnostics. At present, most of such determinations use antibodies - either polyclonal or monoclonal - as the biospecific binding partner. The determinations that use antibodies are often called immunoassays. Immunoassays are often divided into non-competitive and competitive ones, where non-competitive assays involve the use of an excess of reagents and two biospecific binding partners binding to the same analyte (this type of assay is commonly called the sandwich assay). Competitive assays on the other hand rely on the measurement of the ratio between the free and bound labelled marker, with the ratio being modified by the amount of the analyte in the sample.

In usual non-competitive immunoassays, a sample containing the antigen to be determined is incubated with an excess of a capture antibody immobilized to a solid support. A labelled antibody, specific for another epitope on the same antigen, is added in excess. A sandwich comprising "catching antibody - antigen - labelled antibody" is thus formed. Aft r completion of the incubation, the unbound labelled antibody is removed and the signal from the label

in the sandwich is measur d. The signal is thus directly proportional to the antigen concentration in the sampl .

The above non-competitive method cannot, however, be easily applied to the determination of small molecular weight analytes. The small molecular weight analyte is too small to simultaneously bind to two different antibodies. The immunoassay of these analytes is therefore normally performed by a competitive assay. In a typical implementation of competitive assay, the sample containing the analyte to be determined as well as a labelled derivative of the analyte are added to an immobilized antibody specific for said analyte. The unlabelled and labelled analytes compete for the binding sites on the antibody. After completion of the incubation, unbound analytes are removed and the signal from the bound labelled analyte is detected. Increased concentrations of the analyte in the sample will thus result in a decreased signal. When the signal strength is plotted as a function of increasing analyte concentration, a sigmoidal, descending curve is obtained. The sensitivity of this competitive assay is not as high as that of non-competitive assays due to the fact that the signal is at its highest value at the zero dose. As Ekins et al. [1,2] have pointed out, sensitivity can be defined as the smallest dose distinguishable from the zero dose. A usual criterion for this distinction is that the signal of the dose differs by more that two standard deviations (SD) from the signal of the zero dose. In a competitive assay one has to detect a small difference between two high signals, whereas in a non-competitive assay one has to detect a small difference between two low signals. As the SD of the low signal tends to be less than that of the high signal, the non-competitive assay is able to detect smaller differences in signal than the competitive assay. Assuming that the slopes of the dose-response curves of both assays have the same absolute value in the low dose range, then non-competitive assay is more sensitive, because its ability to detect

smaller differ nces in signal is in direct proportion to its ability to detect smaller differences in the dose, too.

We have recently discovered a new non-competitive method for the determination of small molecular weight analytes such as haptens. Contrary to the usually employed competitive assay described above, the new method gives a linear, ascending curve when signal strength is plotted as a function of increasing analyte concentration. This key feature of the new method makes it more sensitive than the competitive immunoassay.

SUMMARY OF THE INVENTION

This invention relates to a non-competitive method for the determination of analytes, comprising the steps of
a) contacting a sample containing the analyte with a binding partner specific for said analyte,
b) adding a blocker, said blocker being able to inactivate the binding sites of said binding partner that are unoccupied, but not being able to inactivate the binding sites of said binding partner that are occupied by the analyte,
c) dissociating the bound analyte from the binding partner,
d) adding a labelled marker which is able to occupy the binding sites from which the analyte was dissociated, but which is not able to occupy the binding sites that were inactivated by the blocker, wherein step d can be performed subsequently to or simultaneously with step c, and
e) measuring the signal from the labelled marker bound to the binding partner.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A shows the signal strength versus concentration of 17ß-Estradiol in the range 0 to 0.5 nM measured according to the method of this invention.

Figure 1B shows the signal strength versus concentration of 17ß-Estradiol in th range 0 to 2 nM measur d according to th method of this invention.

In both figures the filled squares represent replacement time of 10 minutes and the open squares represent replacement time of 5 minutes.

DETAILED DESCRIPTION OF THE INVENTION

In the context of this invention the term "analyte" shall mean any molecule for which there exists a specific binding partner. The present invention is especially suited for the determination small (i.e. molecular weight less than 5000 Daltons) analytes. Such analytes are common among the groups like steroids, vitamins, prostaglandins, antiasthmatic drugs, antiarrythmic drugs, antineoplastic drugs, anticonvulsant drugs, antibiotics, antiarthritic drugs, antidepressant drugs, and drugs of abuse such as cocaine, morphine, heroin, amphetamine, methamphetamine, cannabinoids and the like, and environmental pollutants and toxins.

The "binding partner" is defined as an entity which has sufficient affinity and specificity for the analyte. Typical binding partners are macromolecules such as proteins and nucleic acids.

Proteins which are particularly suitable as binding partners include antibodies and receptors. Antibodies may be raised in animals [3] or they may be selected from recombinant libraries [4]. Antibodies specific for important analytes are commercially available from various sources. Receptors are naturally present in humans and other organisms, and they may be isolated from these sources. Alternatively, the genes coding for th receptors may be cloned by recombinant DNA technology [5], transferred to appropriat host organisms and expressed

there to produce the receptor of interest [5,6]. Other naturally occurring proteins which may be suitable as binding partners ar various carrier and transporting proteins, e.g. sex hormone binding globulin (SHBG). If no suitable binding partner is found among naturally existing proteins, one may be created by protein engineering, either *de novo* or using an existing protein as a starting material. Such protein engineering would involve either genetic engineering or chemical modifications to the protein or both.

The term "blocker" shall mean any substance that prevents the unoccupied binding partner from binding the labelled marker, but which does not prevent the occupied binding partner from liberating the analyte bound thereto and subsequently binding the labelled marker.

Preferably the blocker is a molecule exhibiting the same or similar epitope as the analyte to be determined, resulting in a mutually exclusive binding of the analyte and the blocker to the binding partner. The blocker is thus preferably, but not necessarily, a derivative of the analyte.

The blocker may also be an antibody that binds to the binding partner only when the analyte is not bound to the binding partner and which antibody, when bound to the binding partner, prevents the labelled marker from binding to the binding partner.

The blocker may also be a nucleic acid (DNA or RNA) that binds to the binding partner only when the analyte is not bound to the binding partner and which nucleic acid, when bound to the binding partner, prevents the labelled marker from binding to the binding partner.

Further, the blocker may also be a substance that modifies the unoccupied binding partner in such a fashion that it is

no more able to bind the labelled marker, but which substance does not modify the occupied binding partner in such a fashion that it would not be abl to liberate th analyte bound thereto and subsequently bind the labelled marker. The modifying substance may be a chemical compound or an enzyme.

A blocker that is a chemical compound may modify the binding partner by reacting with a specific residue in the binding partner. Such reactions are for example alkylation of a free cysteine residue by a maleimide derivative or a iodoacetate derivative, nitration of a tyrosine residue by tetranitromethane, bromination of a tryptophan residue by N-bromosuccinimide or the iodination of a tryptophan residue by iodine. These examples of chemical modification of the binding partner are well known to the skilled art worker, and are widely reviewed in the literature of the art. A general but not exhaustive description of these techniques is presented in reference [7]. Further, the chemical compound may be a reactive derivative of the analyte such as an aryl derivative [7], which binds to the binding partner first by biospecific recognition, and later forms a covalent bond by virtue of the reactive group. If the reactive group is an arylazide, the formation of the covalent bond can be controlled by photoactivation with ultraviolet or visible light [8].

A blocker that is an enzyme may modify the binding partner for example by digestion, by adding a phosphate, by removing a phosphate, by glycosylation or by deglycosylation. Enzymes that digest the binding partner are for example proteases, such as trypsin, pepsin, papain, factor Xa, V8 or enterokinase. Further, the enzymes that digest the binding partner may also be nucleases, if the binding partner or a part thereof is a nucleic acid (DNA or RNA). Such nucleases include for example type II restriction endonucleases, exonuclease III, DNAse I, and RNAses. Enzymes that add a phosphate are for example

kinas s such as a tyrosin kinase or a serine kinase. Enzymes that remove a phosphate are for example phosphatases such as LAR prot in tyrosine phosphatase. Enzymes that add or remove a glycosyl group are for example glycosylases or glycosidases.

If the blocker is a molecule that binds non-covalently to the binding partner, its rate of dissociation from the binding partner must be lower than that of the analyte (at least five times as low, preferably more than one hundred times as low, most preferably more that one thousand times as low).

If the blocker is a chemical compound or an enzyme, it must be able to modify most of the unoccupied binding partners (at least more than 90%, preferably more than 95%, typically more than 99%, most preferably more than 99.8%).

The term "inactivate" shall mean any of the above listed mechanisms whereby the blocker prevents the unoccupied binding partner from binding the labelled marker.

The "marker" is a molecule which is able to bind to the binding partner only when the binding partner is not occupied by the analyte and when it is not inactivated by the blocker. Preferably, but not necessarily, the marker and the analyte bind mutually exclusively to the same binding site on the binding partner. The marker is either labelled before the replacement reaction, or it can be labelled after the replacement reaction. The label can be for example a radio-isotope, an enzyme or a fluorescent, phosphorescent or luminescent molecule.

<u>Preferred embodiments</u>

The main steps of a preferred embodiment of the method according to th invention are illustrated on Scheme 1.

The m thod according to this invention is particularly suitabl for the m asurement of 17ß-estradiol. In one such embodiment the analyt is 17ß-estradiol, the binding partner is for example a monoclonal antibody specific for 17ß-estradiol, the blocker is for example 17ß-estradiol-6-carboxymethyl oxime, and the labelled marker is for example Eu-labelled 17ß-estradiol-6-carboxymethyl oxime.

Although a solid phase-bound antibody is used in the examples below, the invention is not limited to this kind of embodiment. Equally well other embodiments could be devised in which the incubation of the analyte with the binding partner could take place in solution.

The invention is illuminated by the following examples.

Example 1

Préparation of Europium-labelled 17ß-Estradiol-6-carboxymethyl oxime.

Europium chelate of 4-[2-(4-aminophenyl)ethynyl]-2,6-bis{[N,N-bis(carboxymethyl)-amino]methyl}pyridine was a gift from H. Mikola, Wallac Oy. It was synthetized by the method of Takalo *et al.* [9,10]. 6-oxoestradiol 6[O-(6-aminohexyl)oxime] (abbreviated 6-AHO) was a gift from H. Mikola, Wallac Oy. It was prepared according to the method of Mikola and Hänninen [11]. Europium-labelled estradiol was a gift from H. Mikola, Wallac Oy. It was synthetized from 6-AHO and the Europium chelate of 4-[2-(4-aminophenyl)ethynyl]-2,6-bis{[N,N-bis(carboxymethyl)-amino]methyl}pyridine using water soluble carbodiimide as condensation agent in a buffer-dioxane solution and purified on a gel filtration column using methods of Mikola and Miettinen [12] and Mikola *et al.* [13].

Example 2

Determination of affinity constants (Ka) of various monoclonal antibodies for Europium-labelled 17ß-Estradiol-6-carboxymethyl oxime

Three commercially available monoclonal anti-17ß-estradiol antibodies were tested. These antibodies were all raised against position 6 derivatives of 17ß-Estradiol. The monoclonal antibodies used were 069-A5406A (BiosPacific, California), 10-E15 (Fitzergald Industries International Inc., Massachusetts) and 2F9 (InterPharm Laboratories Ltd., Israel) and they had been raised against the following immunogens:

Antibody	Immunogen (According to manufacturer)
069-A5406A	Estradiol-6-Bovine serum albumin
10-E15	Estradiol-6-Carboxymethyloxime-carrier
2F9	Estradiol-6-Carboxymethyloxime-Bovine serum albumin

The affinity constants of the monoclonal antibodies 069-A5406A, 10-E15 and 2F9 for Europium-labelled 17ß-Estradiol-6-carboxymethyl oxime (see Example 1) were determined as follows:

All incubations were performed at 22 °C. Antibodies were diluted in Assay buffer [50 mM Tris-HCl pH 7.75, 0.9% NaCl, 0.05% NaN_3, 0.01% Tween 40, 0.05% Bovine gammaglobulin, 20 µM Diethylenetriaminepentaacetic acid, 0.5% Bovine serum albumin, 20 µg/ml Cherry Red] at a concentration of 10 ng/ml. The diluted antibodies were placed in the wells of rabbit anti-Mouse IgG-coated microtitration strips (Wallac Oy, Turku, Finland), 200 µl per well, and the strips were shaken in a plate shaker at 600 rounds per minute for 2 hours. During the incubations, serial dilutions of Europium-labelled estradiol wer mad . After the incubations, the strips were wash d four times with washing

solution [0.9% (w/v) NaCl, 5 mM Tris-HCl pH 7.75, 0.005% Tween]. 200 µl of each dilution of the Europium-labelled estradiol were added to separate wells and the strips were shaken in a plate shaker at 600 rounds per minute for 2 hours. The strips were washed four times with washing solution, after which 200 µl of Delfia Enhancement solution (Wallac) was added. After an incubation of 30 min the time-resolved fluorescence signals were read with a Plate fluorometer (Wallac). The data were plotted as bound/free vs. bound Europium-labelled estradiol and the affinity constants were calculated from the slopes of the plots according to the method of Scatchard [14].

Results:

Affinity constants (Ka (L/mol)) for complexes between Eu-labelled 17ß-Estradiol-6-carboxymethyl oxime and the various monoclonal antibodies.

Mab	Ka
069-A5406A	6.7×10^{10}
10-E15	2.5×10^{10}
2F9	9.4×10^{10}

Example 3

Determination of half-lives of complexes between various monoclonal antibodies and 17ß-Estradiol, 17ß-Estradiol-6-carboxymethyl oxime or 17ß-Estradiol-6-aminohexyl oxime

The half-lives of complexes between monoclonal antibodies (069-A5406A, 10-E15 or 2F9) and 17ß-Estradiol (Sigma Chemical Company), 17ß-Estradiol-6-carboxymethyl oxime (abbreviated 6-CMO; Sigma Chemical Company) or 17ß-Estradiol-6-aminohexyl oxime (abbreviated 6-AHO, see Example 1) were determined as follows:

All incubations were performed at 22 °C; all dilutions were done in Assay buffer [50 mM Tris-HCl pH 7.75, 0.9% NaCl, 0.05% NaN_3, 0.01% Tween 40, 0.05% Bovine gammaglobulin, 20 µM Diethylenetriaminepentaacetic acid, 0.5% Bovine serum albumin, 20 µg/ml Cherry Red]. Diluted antibodies (10 ng/ml) were placed in the wells of rabbit anti-Mouse IgG-coated microtitration strips (Wallac), 200 µl per well, and the strips were shaken in a plate shaker at 600 rounds per minute for 2 hours. The strips were washed four times with washing solution [0.9% (w/v) NaCl, 5 mM Tris-HCl pH 7.75, 0.005% Tween], after which 200 µl of either 100 nM 17ß-estradiol, 10 nM 6-CMO or 10 nM 6-AHO were added to the wells and the strips were shaken in a plate shaker at 600 rounds per minute for 60 min. The strips were washed four times with washing solution, after which 200 µl of 10 nM Europium-labelled 17ß-Estradiol was added. The strips were again shaken in the plate shaker at 600 rounds per minute for varying times. The strips were taken from the plate shaker one at a time and washed four times with the washing solution, after which Delfia Enhancement solution (200 µl per well; Wallac) was added. After the addition of the enhancement solution, each strip was incubated for 30 min before the time-resolved fluorescence signals were read with a Plate fluorometer (Wallac). The data were plotted as the natural logarithm of the signal (cps) vs. the elapsed time in minutes (from the addition of Europium-labelled estradiol to the start of the washes). Half-lifes were calculated from the slopes of the plots with the following formula:

Half-life (min) = [ln(2)]/-k , where k is the slope of the plot.

Results:

Half-lives (in minutes) of Mab-antigen complexes with the antiges 17β-Estradiol, 6-CMO and 6-AHO.

Mab	Antigen		
	17ß-Estradiol	6-CMO	6-AHO
069-A5406A	15	47	64
10-E15	9	127	93
2F9	44	51	154

For the purpose of this invention the Mab 10-E15 seemed most promising because there is a 14-fold difference between the half-lives of its complexes with 17ß-Estradiol (9 min) and 6-CMO (127 min).

Example 4

Production of a dose-response curve for 17ß-Estradiol

All incubations were performed at 22 °C; all dilutions were done in Assay buffer [50 mM Tris-HCl pH 7.75, 0.9% NaCl, 0.05% NaN_3, 0.01% Tween 40, 0.05% Bovine gammaglobulin, 20 µM Diethylenetriaminepentaacetic acid, 0.5% Bovine serum albumin, 20 µg/ml Cherry Red]. Mab 10-E15 (Fitzgerald Industries International Inc., Massachusetts) was immobilized in the wells of rabbit anti-Mouse IgG-coated microtitration strips (Wallac Oy, Turku, Finland), by adding 100 µl per well of a 100 ng/ml dilution of the antibody, shaking in a plate shaker at 600 rounds per minute for 1 hour, and washing the strips four times with washing solution [0.9% (w/v) NaCl, 5 mM Tris-HCl pH 7.75, 0.005% Tween]. 100 µl per well of 17ß-Estradiol standards of the concentrations 0, 0.025, 0.050, 0.075, 0.1, 0.2, 0.5 and 2 nM were added to the strips containing the immobilized antibody, after which the strips were shaken in the plate shaker at 600 rounds per minute for 2 hours. The blocking reactions were initiated by adding 100 µl per well of a 10 µM solution of 17ß- stradiol-6-carboxymethyl oxime

(Sigma Chemical Company). The strips were further shaken for 5 min and then washed with washing solution four times. The replacement reaction was initiat d by adding 100 µl per well of 10 nM Europium-labelled 17ß-estradiol, after which the strips were shaken for either 5 or 10 minutes and washed with washing solution four times. 200 µl of Delfia Enhancement solution (Wallac) was added and the plates were shaken for 30 min after which the time-resolved fluorescence signal was read with the Plate Fluorometer (Wallac).

Dose-response curves were prepared by plotting the signals against the concentrations of the standards in an X-Y plot, see Figure 1A and 1B. The filled squares represent replacement time of 10 minutes and the open squares represent replacement time of 5 minutes. Figure 1A shows the curves in the concentration range 0 to 0.5 nM and Figure 1B shows the curves over the whole measuring range of 17ß-Estradiol standard concentrations. Every point shown is an average of three parallel determinations. Error bars extend ± 1 SD of the mean value in each point.

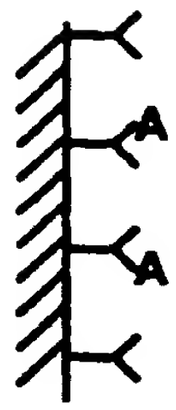
The least detectable dose LDD was defined as the 17ß-Estradiol concentration which gives a signal that differs by two standard deviations from the zero-concentration signal. This value is calculated by dividing the standard deviation (SD) of the zero concentration signal by the slope of the linear portion of the standard curve.

It will be appreciated that the methods of the presented invention can be incorporated in the form of a variety of embodiments, only a few of which are disclosed herein. It will be apparent to the specialist that other embodiments exist and do not depart from the spirit of the invention. Thus, the described embodiments are only illustrative and should not be construed as restrictive.

SCHEME 1

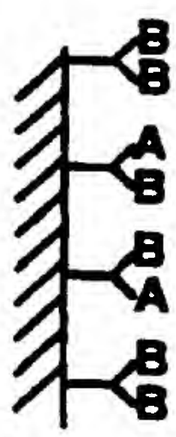
1. Add sample

- A = antigen in sample
- coated antibody in excess => A binds quantitatively



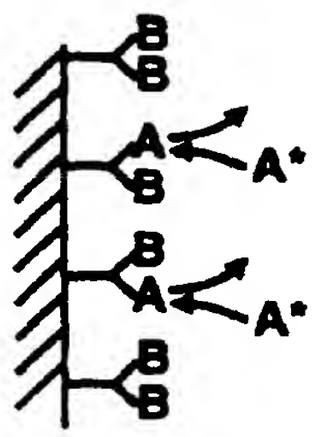
2. Add blocker

- B = blocker = tight binding analog to antigen A
- excess B fills all empty sites
- wash unbound B away



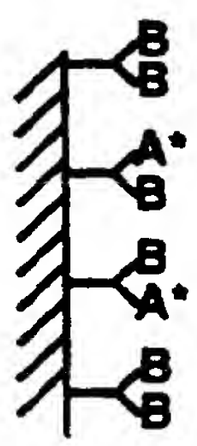
3. Add labelled marker

- A* = labelled marker (antigen)
- excess A* will replace A
- tight-binding B will not be replaced
- wash unbound A* away



4. Measure signal

- signal is directly proportional to original A in sample
- will give an ascending standard curve
- is more sensitive than competitive assay



LIST OF REFERENCES

1. Ekins, R.P. and Chu, F.W. (1991) Multianalyte microspot assay - microanalytical "compact disk" of the future. Clin. Chem. 37:1955-67.

2. Ekins, R.P. and Dakubu, S. (1985) The development of high sensitivity pulsed light, time-resolved fluoroimmunoassays. Pure & Appl. Chem. 57: 473-82.

3. Harlow, E. and Lane, D. (1988) Antibodies: a laboratory manual. Cold Harbor Laboratory, Cold Spring Harbor, New York, U.S.A.

4. Winter, G., Griffiths, A.D., Hawkins, R.E. and Hoogenboom, H. (1994) Making antibodies by phage display technology. Annu. Rev. Immunol. 12: 433-55.

5. Sambrook, J. Fritch, E.F. and Maniatis. T. (1989) Molecular cloning: a laboratory manual. 2nd edition. Cold Spring Harbor Laboratory Press. Cold Harbor Laboratory, Cold Spring Harbor, New York, U.S.A.

6. Walker, J.M. and Gingold, E.B., eds. (1993) Molecular biology and biotechnology. 3rd edition. The Bath Press, Bath, UK.

7. Imoto, T. and Yamada, H. (1989) Chemical modification. In Creighton, T.E. (ed.) Protein function. A practical approach. IRL press, Oxford, England.

8. Pierce Catalog and Handbook, Pierce, Rockford, Illinois, U.S.A.

9. Takalo, H., Hänninen, E. and Kankare, J. (1993) Luminescence of Europium (III) chelates with 4-(arylethynyl)pyridines as ligands. Helv. Chim. Acta 76: 877-83.

10. Takalo, H., Mukkala, V.-M., Mikola, H., Liitti, P. and Hemmilä, I. (1993) Synthesis of Europium(III) chelates suitable for labeling of bioactive molecules. Bioconjugate Chem. 5: 278-82.

11. Mikola, H. and Hänninen, E. (1992) Introduction of aliphatic amino and hydroxy groups to keto steroids using O-substituted hydroxylamines. Bioconjugate Chem. 3: 182-6.

12. Mikola, H. and Miettinen, P. (1991) Preparation of Europium labeled derivatives of cortisol for time-resolved fluoroimmunoassays. Steroids 56: 17-21.

13. Mikola, H., Sundell, A.-C. and Hänninen, E. (1993) Labeling of estradiol and testosterone alkyloxime derivatives with a europium chelate for time-resolved fluoroimmunoassays. Steroids 58: 17-21.

14. Scatchard, G. (1949) The attraction of proteins for small molecules and ions. Ann. NY. Acad Sci. 51:660-672.

15. Mikola, H. and Hänninen, E. (1992) Introduction of aliphatic amino and hydroxy groups to keto steroids using O-substituted hydroxylamines. Bioconjugate Chem. 3: 182-6.

CLAIMS

1. 1. A method for the determination of analytes comprising the steps of

a) contacting a sample containing said analyte with a binding partner specific for said analyte,

b) adding a blocker, said blocker being able to inactivate the binding sites of said binding partner that are unoccupied, but not being able to inactivate the binding sites of said binding partner that are occupied by the analyte,

c) dissociating the bound analyte from the binding partner,

d) adding a labelled marker which is able to occupy the binding sites from which the analyte was dissociated, but which is not able to occupy the binding sites that were inactivated by the blocker, wherein step d can be performed subsequently to or simultaneously with step c, and

e) measuring the signal from the labelled marker bound to the binding partner

2. A method according to claim 1 wherein the binding partner is an antibody.

3. A method according to claim 1 wherein the binding partner is immobilized to a solid phase.

4. A method according to claim 1 wherein the blocker is bound to the binding partner by specific molecular recognition.

5. A method according to claim 4 wherein the blocker is non-covalently bound to the binding partner, and the rate

of dissociation of th block r from the binding partner is at l ast five times lower than the rate of dissociation of th analyte from the binding partner.

6. A method according to claim 4 wherein the blocker is covalently bound to the binding partner by virtue of a reactive group present in the blocker.

7. A method according to claim 1 wherein the blocker is a substance that chemically or enzymatically modifies the binding partner so as to inactivate its binding site.

9. A method according to any of the foregoing claims wherein the analyte is a steroid.

10. A method according to claim 9 wherein the analyte is 17ß-estradiol, the blocker is 17ß-estradiol-6-carboxymethyl oxime, and the labelled marker is labelled 17ß-estradiol-6-carboxymethyl oxime.

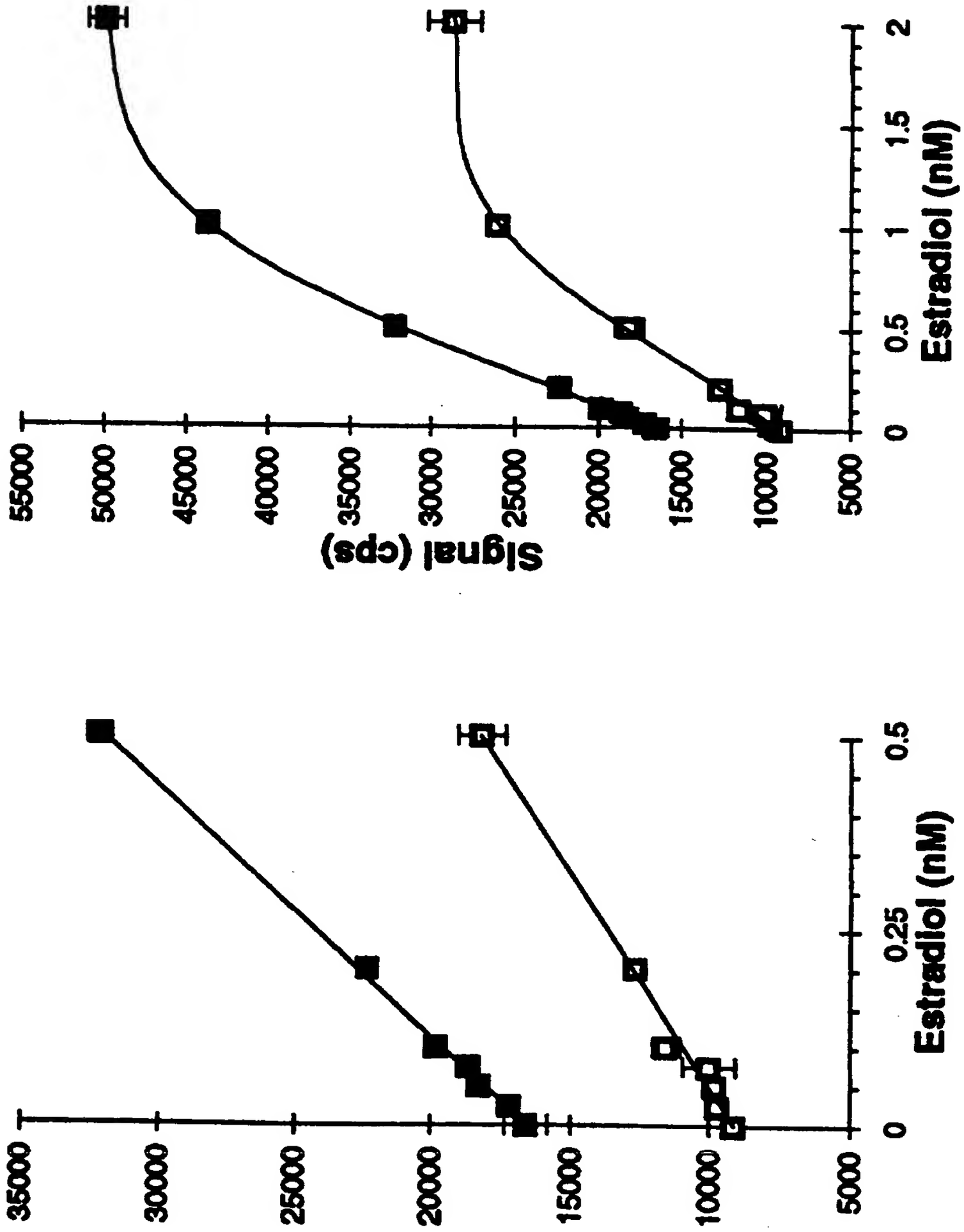

Signal (cps)
55000
50000
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
0.5
1
1.5
2
Estradiol (nM)

FIG. 1B

Signal (cps)
35000
30000
25000
20000
15000
10000
5000
0
0.25
0.5
Estradiol (nM)

FIG. 1A

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI 97/00059

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: G01N 33/543, G01N 33/53
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5476770 A (PHILIPPE PRADELLES), 19 December 1995 (19.12.95) --	1
A	Anal. Chem., Volume 66, 1994, P. Pradelles et al, "Immunometric Assay of Low Molecular Weight Haptens containing Primary Amino Groups" page 16 - page 22 --	1
A	Patent Abstracts of Japan, Vol 4,No 142, P-30, abstract of JP,A,55-90858 (ASAHI KASEI KOGYO K.K.), 9 July 1980 (09.07.80) --	1

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 May 1997	25 -05- 1997
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Carl Olof Gustafsson Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI 97/00059

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 8905453 A1 (CAMBRIDGE PATENT DEVELOPMENTS LIMITED), 15 June 1989 (15.06.89), see page 3-5 -- --------	1

INTERNATIONAL SEARCH REPORT

Information on patent family members

02/04/97

International application No.
PCT/FI 97/00059

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5476770 A	19/12/95	CA 2113383 A	29/07/94
		EP 0609144 A	03/08/94
		FR 2700855 A,B	29/07/94
		JP 6273416 A	30/09/94
WO 8905453 A1	15/06/89	AT 108907 T	15/08/94
		AU 2726388 A	05/07/89
		DE 3850784 D,T	09/03/95
		EP 0396570 A,B	14/11/90
		GB 2214295 A,B	31/08/89
		US 5468651 A	21/11/95